REMARKS

Claim Status

Claims 1-18 are pending. Applicants have canceled claim 2 without prejudice to pursue the subject matter in a future application. Claims 1, 3, 12, 14, 15 and 16 have been amended.

Support for the amendments to the claims

Support for amended claim 1 may be found *inter alia* on page 17 of the Specification, lines 15-27; claim 1 and claim 2 as filed.

Support for amended claim 3 may be found *inter alia* on page 17 of the Specification, lines 21-24.

Support for amended claim 12 may be found *inter alia* on page 5 of the Specification, line 10.

Support for amended claims 13-16 may be found *inter alia* in claims 13-16 as filed.

Applicants respectfully request the entry of this Amendment. Upon entry, Claims 1, 3-18 are pending and under examination.

Rejections Under 35 U.S.C. §112

Claims 12-13 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite. The rejection is respectfully traversed.

Claim 12 has been amended to recite that the tissue animal cells are brain neurons. Claim 13 has been amended to clarify that the aim is to express the sequences in an animal cell. Applicants submit that claim 12 and 13 have been amended to obviate the rejection. Accordingly, Applicants respectfully

request that the rejection of claims 12-13 under 35 U.S.C. §112, second paragraph, be withdrawn.

Rejections Under 35 U.S.C. §102

Claims 1-4 and 6-15 are rejected under 35 U.S.C. §102(e) as being anticipated by Chtarto et al. The rejection is respectfully traversed.

Chtarto et al. is directed exclusively to a vector comprising a bi-directional antibiotic controlled activator-responsive promoter. In particular, the promoter comprises a tetracycline (Tet) responsive element.

The **Tet responsive promoter** is a synthetic sequence composed of 7 repetitions of an 8 nucleotide-long **prokaryotic sequence.** Such promoter is not able to exploit the endogenous mammalian transcriptional machinery in order to work properly. Therefore, to render this promoter functional, it is necessary to express an **additional** transgene encoding for a chimeric transcriptional activator composed of two halves, the first one being of **prokaryotic** origin and the second one being of **viral or human** origin.

A fundamental feature of the tetracycline-responsive expression system is that the promoters used must be insulated from nearby competing enhancers in order to prevent inappropriate transactivation and preserve integrity of modulation by doxycycline. Therefore, only minimal promoters have been so far used in this system. Moreover, the Tet-regulated system depends on the expression (or exogenous administration) of transactivators, whose expression (or administration) may encounter problems in vivo.

In contrast, the present invention is directed to **synthetic eukaryotic bidirectional promoters**, based on the juxtaposition of a core promoter element placed upstream and in opposite orientation to an efficient promoter, that exploits the endogenous transcriptional machinery available to most animal cells types to drive robust expression of two divergent transcripts. The fact that the bidirectional promoter of the present invention comprises a minimal **viral** promoter **and** a **full length animal** promoter represents a fundamental difference over the prior art in general, and over Chtarto et al. in particular.

It is well known in this art that the eukaryotic and prokaryotes promoters act differently. For example,

> *The promoter contains specific DNA sequences, response elements, that are recognized by proteins known as transcription factors. These factors bind to the promoter sequences, recruiting RNA polymerase, the enzyme that synthesizes the RNA from the coding region of the gene.*
>
> - *In prokaryotes, the promoter is recognized by RNA polymerase and an associated sigma factor, which in turn are brought to the promoter DNA by an activator protein binding to its own DNA sequence nearby.*
> - *In eukaryotes, the process is more complicated, and at least seven different factors are necessary for the transcription of an RNA polymerase II promoter."*

See **Exhibit A, 5 pages.**

In addition, it is well known from general biology text books that a promoter of a prokaryote organism cannot work in the environment of an eukaryote organism and vice versa.

The bidirectional promoter of the present invention can be based on **any eukaryotic promoter**: ubiquitous, constitutive, tissue specific or endogenously regulated.

In nature, few instances of bidirectional promoters have been documented and only very recently recent surveys of the human genome have indicated an abundance of divergently transcribed gene pairs representing more than 10% of the human genome, whose transcriptional start sites are separated by less than 1 kb. In addition, it has been suggested that more than half of the human promoters does not exhibit strong directionality in transcript initiation and can function in a bidirectional fashion. Thus, the synthetic bidirectional promoters of the present invention may mimic a well-represented and evolutionary conserved feature of eukaryotic transcription, providing a structural basis for their robust performance.

More specifically, Chtarto et al. require the use of a transactivator factor encoded by a reverse antibiotic controlled transactivator nucleotide sequence for activating the bi-directional promoter. *See*, for instance, Chtarto et al. column 5, lines 12-16:

> *In said construct or system the bi-directional antibiotic controller activator responsive promoter/operator sequence 4 is advantageously activated by the transactivator factor 7, encoded by the reverse antibiotic controlled transactivator 7, encoded by the reverse antibiotic controlled transactivator nucleotide sequence 6 in the presence of said antibiotic 5.*

That Chtarto et al. is only directed to an antibiotic inducible/repressible genetic construct is clear throughout the document. *See* further Fig. 6, column 4, lines 42-43, column 5, lines 22-32.

The present invention offers the construction of a bidirectional promoter comprising a minimal viral promoter and a full length eukaryotic promoter. *See*, for instance, description page 7, lines 3-8:

A bidirectional promoter made by minimal core promoter elements from the human cytomegalovirus (mCMV) joined upstream, and in opposite orientation, to an efficient promoter, derived from the human phosphoglycerate kinase (PGK) or poly-ubiquitin UBI-C gene, was driving divergent transcription of two RNAs.

In conclusion, Chtarto et al. do not anticipate the present invention because Charto et al. do not teach each and every aspect of the present invention. Accordingly, Applicants respectfully request that the rejection of claims 1-4 and 6-15 under 35 U.S.C. §102(e) be withdrawn.

Claims 1-4 and 6-11 and 13-18 are rejected under 35 U.S.C. §102(e) as being anticipated by Itoh et al. The rejection is respectfully traversed.

Itoh et al. is directed exclusively to a vector comprising a low molecular weight compound-responsive bidirectional promoter and a DNA encoding a low molecular weight compound-controlled transactivator. In particular, the low molecular weight compound is tetracycline or doxycycline and the low molecular weight compound-controlled transactivator is a reverse tetracycline transactivator.

Therefore, arguments presented above concerning Chtarto et al. are also valid in respect to Itoh et al.

In conclusion, Itoh et al. do not anticipate the present invention because Itoh et al. do not teach each and every aspect of the present invention. Accordingly, Applicants respectfully request that the rejection of claims 1-4 and 6-11 and 13-18 under 35 U.S.C. §102(e) be withdrawn.

Claims 1-4, 7-8, 10 and 14 are rejected under 35 U.S.C. §102(b) as being anticipated by Fux et al. The rejection is respectfully traversed.

Fux et al. discloses the construction of two vectors, PDuoRex7 and pDuoRex8 (*see* page 114, left column, last paragraph) which contain two antibiotic-responsive expression units in divergent orientation. Such pDuoRex-based dual regulated expression requires concomitant production of tTA and PIT (*see* page 114, right column, first paragraph).

Thus, arguments presented above concerning Chtarto et al. are also valid in respect to Fux et al.

In conclusion, Fux et al. do not anticipate the present invention because Fux et al. do not teach each and every aspect of the present invention. Accordingly, Applicants respectfully request that the rejection of claims 1-4, 7-8, 10 and 14 under 35 U.S.C. §102(b) be withdrawn.

It should be noted that none of the cited prior art documents discloses a bidirectional promoter comprising a minimal viral promoter and a full length animal promoter. Therefore none of the cited documents anticipate the present invention.

Moreover, starting from the teaching of Chtarto et al., or Itoh et al. or Fux et al. and due to the evolutionary distance between prokaryotes and mammalians and to their differences in the transcriptional machinery, the person skilled in the art would not predict and foresee that mammalian promoters could be also exploited for building a bidirectional promoter. Therefore, the invention is not obvious in respect to such cited prior art documents.

Rejections Under 35 U.S.C. §103

Claim 5 is rejected under 35 U.S.C. §103(a) as being unpatentable over Chtarto et al. or Itoh et al., in view of Hope et al. (US 6,136,597).

Chtarto et al. and Itoh et al. have been discussed above. Hope et al. disclose an RNA export element which mediates efficient transport of RNA from the nucleus to the cytoplasm. This RNA export element is referred to as WPRE. WPRE is a post transcriptional regulatory element. Hence, Hope et al. teach using WPRE to increase transgene expression.

Therefore, even though assuming it is appropriate to combine Chtarto et al., and Hope et al., or Itoh et al., and Hope et al., (which the Applicants do not concede), such combination would not teach or suggest all of the features of dependent claim 5. In particular, the combined teaching of Chtarto, Itoh and Hope does not teach or suggest constructing a bidirectional promoter comprising a minimal viral promoter and a full length animal promoter.

Accordingly, Applicants respectfully request that the rejection of claim 5 under 35 U.S.C. §103(a) be withdrawn.

For the Examiner's information, the corresponding **European Application** of this subject application has been granted and is attached hereto as **Exhibit B, 71 pages.**

Conclusion

In summary, Applicants believe that all grounds of rejections have been addressed and earnestly request the Examiner to place this application in condition for allowance.

If a telephone interview would be of assistance in advancing prosecution of the subject application, Applicants' undersigned attorney invites the Examiner to telephone him at the number provided below. If any other fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 50-1891.

Respectfully submitted,

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EXHIBIT A

Promoter

From Wikipedia, the free encyclopedia

In biology, a **promoter** is a regulatory region of DNA located upstream (towards the 3' region of the anti-sense strand) of a gene, providing a control point for regulated gene transcription.

Contents

Overview

The promoter contains specific DNA sequences, response elements, that are recognized by proteins known as transcription factors. These factors bind to the promoter sequences, recruiting RNA polymerase, the enzyme that synthesizes the RNA from the coding region of the gene.

- In prokaryotes, the promoter is recognized by RNA polymerase and an associated sigma factor, which in turn are brought to the promoter DNA by an activator protein binding to its own DNA sequence nearby.

- In eukaryotes, the process is more complicated, and at least seven different factors are necessary for the transcription of an RNA polymerase II promoter.

Promoters represent critical elements that can work in concert with other regulatory regions (enhancers, silencers, boundary elements/insulators) to direct the level of transcription of a given gene.

It is worth noting that promoters are not DNA specific, and can in fact locate upstream towards the 3' end of an RNA genome, e.g. Respiratory Syncytial Virus (RSV).

Identification of relative location

As promoters are typically immediately adjacent to the gene in question, positions in the promoter are designated relative to the transcriptional start site, where transcription of RNA begins for a particular

gene (i.e., positions upstream are negative numbers counting back from -1, for example -100 is a position 100 base pairs upstream).

Promoter elements

- Core promoter - the minimal portion of the promoter required to properly initiate transcription
  - Transcription Start Site (TSS)
  - Approximately -34
  - A binding site for RNA polymerase
    - RNA polymerase I: transcribes genes encoding ribosomal RNA
    - RNA polymerase II: transcribes genes encoding messenger RNA and certain small nuclear RNAs
    - RNA polymerase III: transcribes genes encoding tRNAs and other small RNAs
  - General transcription factor binding sites
- Proximal promoter - the proximal sequence upstream of the gene that tends to contain primary regulatory elements
  - Approximately -250
  - Specific transcription factor binding sites
- Distal promoter - the distal sequence upstream of the gene that may contain additional regulatory elements, often with a weaker influence than the proximal promoter
  - Anything further upstream (but not an enhancer or other regulatory region whose influence is positional/orientation independent)
  - Specific transcription factor binding sites

Prokaryotic promoters

In prokaryotes, the promoter consists of two short sequences at -10 and -35 positions *upstream* from the transcription start site. Sigma factors not only help in enhancing RNAP binding to the promoter but helps RNAP target which genes to transcribe.

- The sequence at -10 is called the Pribnow box, or the -10 element, and usually consists of the six nucleotides TATAAT. The Pribnow box is absolutely essential to start transcription in prokaryotes.
- The other sequence at -35 (the -35 element) usually consists of the six nucleotides TTGACA. Its presence allows a very high transcription rate.
- Both of the above consensus sequences, while conserved on average, are not found intact in most promoters. On average only 3 of the 6 base pairs in each consensus sequence is found in any given promoter. No promoter has been identified to date that has intact consensus sequences at both the -10 and -35; it is thought that this would lead to such tight binding by the sigma factor that the polymerase would be unable to initiate productive transcription.
- Some promoters contain a UP element (consensus sequence 5'-TGNTATAAT-3')upstream of the -35 element; the presence of the -35 element appears to be unimportant for transcription from the UP element-containing promoters.

It should be noted that the above promoter sequences are only recognized by the sigma-70 protein that interacts with the prokaryotic RNA polymerase. Complexes of prokaryotic RNA polymerase with other sigma factors recognize totally different core promoter sequences.

```
 <-- upstream                                                   downstream -->
5'-XXXXXXXPPPPPXXXXXXPPPPPXXXXGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGXXXX-3'
        -35       -10       Gene to be transcribed

(note that the optimal spacing between the -35 and -10 sequences is 17 nt)
```

Probability of occurrence of each nucleotide

```
 for -10 sequence
 T    A    T    A    A    T
77%  76%  60%  61%  56%  82%
```

```
 for -35 sequence
 T    T    G    A    C    A
69%  79%  61%  56%  54%  54%
```

Eukaryotic promoters

Eukaryotic promoters are extremely diverse and are difficult to characterize. They typically lie upstream of the gene and can have regulatory elements several kilobases away from the transcriptional start site. In eukaryotes, the transcriptional complex can cause the DNA to bend back on itself, which allows for placement of regulatory sequences far from the actual site of transcription. Many eukaryotic promoters, but by no means all, contain a TATA box (sequence TATAAA), which in turn binds a TATA binding protein which assists in the formation of the RNA polymerase transcriptional complex.[1] The TATA box typically lies very close to the transcriptional start site (often within 50 bases).

Eukaryotic promoter regulatory sequences typically bind proteins called transcription factors which are involved in the formation of the transcriptional complex. An example is the E-box (sequence CACGTG), which binds transcription factors in the basic-helix-loop-helix (bHLH) family (e.g. BMAL1-Clock, cMyc).[2]

Detection of promoters

A wide variety of algorithms have been developed to facilitate detection of promoters in genomic sequence, and promoter prediction is a common element of many gene prediction methods.

Evolutionary change

A major question in evolutionary biology is how important tinkering with promoter sequences is to evolutionary change, for example, the changes that have occurred in the human lineage after separating from chimps.

Some evolutionary biologists, for example Allan Wilson, have proposed that evolution in promoter or regulatory regions may be more important than changes in coding sequences over such time frames.

Binding

The binding of a promoter sequence (P) to a sigma factor-RNAP complex (R) is a two-step process:

1. R+P ↔ RP(closed). $K = 10^7$
2. RP(closed) → RP(open). $K = 10^{-2}$

Diseases associated with aberrant promoter function

Though OMIM is a major resource for gathering information on the relationship between mutations and natural variation in gene sequence and susceptibility to hundreds of diseases, it requires a sophisticated search strategy to extract those diseases that are associated with defects in transcriptional control where the promoter is believed to have direct involvement.

This is a list of diseases that evidence suggests have some involvement of promoter malfunction, either through direct mutation of a promoter sequence or mutation in a transcription factor or transcriptional co-activator.

Keep in mind that most diseases are heterogeneous in etiology, meaning that one "disease" is often many different diseases at the molecular level, though the symptoms exhibited and the response to treatment might be identical. How diseases respond differently to treatment as a result of differences in the underlying molecular origins is partially addressed by the discipline of pharmacogenomics.

Not listed here are the many kinds of cancers that involve aberrant changes in transcriptional regulation owing to the creation of chimeric genes through pathological chromosomal translocation.

Canonical sequences and wild-type

The usage of canonical sequence for a promoter is often problematic, and can lead to misunderstandings about promoter sequences. Canonical implies perfect, in some sense.

In the case of a transcription factor binding site, then there may be a single sequence which binds the protein most strongly under specified cellular conditions. This might be called canonical.

However, natural selection may favor less energetic binding as a way of regulating transcriptional output. In this case, we may call the most common sequence in a population, the wild-type sequence. It may not even be the most advantageous sequence to have under prevailing conditions.

Recent evidence also indicates that several genes (including the proto-oncogene c-myc) have G-quadruplex motifs as potential regulatory signals.

Diseases associated with promoter elements

- Asthma[3][4]
- Beta thalassemia[5]
- Rubinstein-Taybi syndrome[6]

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6. ^ Petrij F, Giles RH, Dauwerse HG, Saris JJ, Hennekam RC, Masuno M, Tommerup N, van Ommen GJ, Goodman RH, Peters DJ, et al. (1995). Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. *Nature.* **376** (6538), 348-351. PMID 7630403

External links

- *Directory of computational tools for detecting promoters in sequence data*: BioDirectory (Directory). *BioDirectory*. Oxford Informatics. Retrieved on 2006-12-11.
- ORegAnno - Open Regulatory Annotation Database
- MeSH *Promoter Regions (Genetics)*

- SwitchDB - An online database used to analyze promoters and transcription start sites (TSSs) throughout the human genome.

- Pleiades Promoter Project - a research project with an aim to generate 160 fully characterized, human DNA promoters of less than 4 kb (MiniPromoters) to drive gene expression in defined brain regions of therapeutic interests.

Retrieved from "http://en.wikipedia.org/wiki/Promoter"
Categories: Gene expression
Hidden categories: Articles with unsourced statements since January 2007 | All articles with unsourced statements

EXHIBIT B

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 1 616 012 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
19.12.2007 Bulletin 2007/51

(21) Application number: **04728627.3**

(22) Date of filing: **21.04.2004**

(51) Int Cl.:
C12N 15/79 *(2006.01)*

(86) International application number:
PCT/IT2004/000227

(87) International publication number:
WO 2004/094642 (04.11.2004 Gazette 2004/45)

(54) SYNTHETIC BI-DIRECTIONAL PROMOTERS AND USES THEREOF

SYNTHETISCHE BIDIREKTIONALE PROMOTOREN UND DEREN VERWENDUNGEN

PROMOTEURS BIDIRECTIONNELS DE SYNTHESE, ET LEURS UTILISATIONS

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PL PT RO SE SI SK TR

(30) Priority: **24.04.2003 US 465080 P**

(43) Date of publication of application:
18.01.2006 Bulletin 2006/03

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- **FUX CORNELIA ET AL: "Bidirectional expression units enable streptogramin-adjustable gene expression in mammalian cells." BIOTECHNOLOGY AND BIOENGINEERING. 5 SEP 2003, vol. 83, no. 5, 5 September 2003 (2003-09-05), pages 618-625, XP002303042 ISSN: 0006-3592**
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- **XIE M.; HE Y.; GAN S.: 'Bidirectionalization of polar promoters in plants.' NATURE BIOTECHNOL. vol. 19, July 2001, pages 677 - 679, XP002390685**

EP 1 616 012 B1

Description

[0001] The present invention relates to bidirectional promoters allowing efficient and coordinate expression of two or more genes, to gene transfer vectors containing these promoters, to particles transducing said vectors into a cell, to the use of said vectors for the delivery and expression of multiple genes in target cells, also for gene therapy, and for the manufacturing of medicaments.

TECHNICAL BACKGROUND

[0002] Expression of multiple transgenes within the same target cells is required for several gene transfer and therapy applications[1]. Gene-function studies are best performed by expressing cDNAs together with a marker gene; by this approach, genetically-modified cells can be identified and monitored in vitro and in vivo. Similarly, gene therapy applications can be improved by purification of gene-corrected cells before in vivo administration, taking advantage of coordinate expression of selectable markers. Genetically-modified cells can be amplified ex vivo or in vivo by introducing growth-promoting or drug-resistance genes together with the therapeutic gene, as recently shown by MGMT-mediated selection of transduced Hematopoietic Stem Cells (HSC)[2]; using this approach, the efficacy of gene therapy can be increased, and its application potentially extended to a wide spectrum of diseases [3,4].

[0003] Conversely, genetically-modified cells expressing conditionally cytotoxic genes, together with the therapeutic gene, can be eliminated in vivo, if adverse events occur; this approach is used to control graft-versus-host disease following donor T-lymphocytes infusion to treat leukemia relapse[5]; it may also provide an important safety provision in HSC gene transfer, given the recent occurrence of leukemia related to vector integration in a successful clinical trial of X-linked Severe Combined ImmunoDeficiency[6]. Coordinate expression of more than one transgene is essential when the activity to be reconstituted by gene transfer depends on multiple subunits encoded by different genes, or requires the synergism of separate molecules. For instance, reconstitution of the dopamine biosynthetic pathway in striatal neurons of Parkinson's disease patients requires co-expression of tyrosine hydroxylase with GTP-cyclohydrolase I and/or DOPA decarboxylase[7]; cancer gene therapy may require co-expression of multiple antigens and/or cytokines in antigen-presenting cells for immunotherapy, and of two T-cell receptor chains in T-cells engineered for adoptive transfer[8].

[0004] In spite of such well-recognized needs, reaching coordinate, high-level expression of multiple transgenes in the majority of target cells has been a significant challenge for gene transfer technology. Two different transgenes have been expressed by two separate vectors; yet, only a fraction of target cells was transduced by both vectors and a heterogeneous population of cells was obtained that expressed either one or two genes in different ratios, preventing reliable studies and/or efficacious applications. Alternatively, two or more transgenes have been expressed by different promoters within the same vector[9]; yet, different tissue specificity and mutual interference between promoters often prevented efficient co-expression in the same target cells[10]. Differential splicing generates multiple transcripts from the same promoter, but it has proven difficult to adapt to viral delivery of multiple transgenes[11]. Chimeric polyproteins that self-process co-translationally into separate components have been generated using the self-cleaving peptide of the Foot and Mouth Disease Virus 2A[12, 13]; however, application of this technology to multiple gene transfer has been limited until now because it requires sophisticated engineering, restricts both proteins to the same cellular compartment, and introduces sequence changes that may affect protein activity, stability, and immunogenicity.

[0005] The most satisfactory approach to multiple gene transfer until now has relied on using internal ribosome entry sites (IRES's)[14]. These sequences, identified in viral and cellular transcripts, control translation in a mRNACap-independent manner and, when inserted between two genes in a bicistronic messenger RNA, allow translation of the downstream gene. The authors tested the performance of different IRES's in the context of self-inactivating (SIN) lentiviral vectors (LVs), and found significant limitations of this approach.

[0006] WO 02/064804 describes bi-directional dual promoter complexes that are effective for enhancing transcriptional activity of transgenes in plants.

[0007] The bi-directional promoters of the invention include a modified enhancer region with at least two core promoters on either side of the modified enhancer in a divergent orientation. The application refers to gene expression in plants. In addition, the approach requires the duplication of tandem oriented enhancer sequences in a modified internal region of the construct, to be joined by two identical or homologous minimal promoters on either sides. The instant invention does not require duplication of enhancer or any other sequences in the efficient promoter of the bi-directional construct, nor are need that the core promoters on either sides of it to share at least 30% identity. Finally, tandem duplication may be incompatible with retro/lentiviral delivery.

[0008] US 6,388,170 discloses plant vectors, having bi-directional promoters, comprising a minimal promoter and a common promoter, wherein said minimal promoters is operably linked to said common promoter, in opposite orientation to said common promoter, and 5' to said common promoter. Promoter sequences derived from plants and plant-infecting viruses are disclosed dnd tested in plant cells or plant parts.

[0009] Given the substantial evolutionary distance between plants and animals, US 6,388,170 does not teach how to

engineer animal promoters for bidirectional activity and whether bi-directional promoters may effectively work in animal cells. In addition, US 6,388,170 does not teach how to engineer bi-directional promoters for gene expression in animals and in animal cells using the available gene transfer methods.

[0010] WO01/34825 discloses cell lines, plasmids and vectors useful for the production of recombinant viruses such as adenoviruses, which are useful in gene therapy. The cell lines, plasmids and vectors comprise inducible promoters, such as bi-directional promoters for the coordinate expression of bidirectionally cloned gene. However only bi-directional Tet-regulated constructs are disclosed.

[0011] Thus, the authors explored novel strategies to take full advantage of gene transfer systems, such as LV, that allow efficient ex vivo transduction and direct in vivo administration.

DESCRIPTION OF THE INVENTION

[0012] The authors developed a novel vector design in which synthetic bi-directional promoters mediated coordinate transcription of two divergent RNAs. The authors show that LVs carrying bi-directional promoters coordinately expressed two transgenes in the vast majority of transduced cells clearly outperforming the bicistronic vectors. The efficient performance of the new bi-directional LVs in primary hematopoietic cells, assayed ex vivo and after transplantation, and in several tissues in vivo, after direct vector delivery or transgenesis was established. The invention overcomes a long-standing hurdle in the quest for improved gene-expression tools and are expected to advance the reach and safety of gene therapy.

[0013] It is therefore an object of the instant invention a bidirectional promoter for expression of at least two coding sequences in opposite direction in animal cells comprising 5' end to 3' end:

a) a first minimal promoter sequence derived from cytomegalovirus (CMV) or mouse mammary tumor virus (MMTV) genomes;
b) a full efficient promoter sequence derived from an animal gene;

the two promoter sequences driving a coordinate transcription of said coding sequences in the opposite orientation.

[0014] In the ambit of the instant invention a full efficient promoter sequence means a sequence driving an efficient transcription of primary transcript. Preferably it comprises an enhancer region and a minimal promoter sequence, either distinct or overlapping. More preferably the full efficient promoter sequence derives from the phosphoglycerate kinase or from the ubiquitin promoter.

[0015] It is an object of the invention a bidirectional expression cassette essentially comprising the bidirectional promoter as above disclosed, convenient insertion sites positioned downstream to each promoter, and polyadenylation sites positioned downstream to each insertion site.

[0016] Preferably the bidirectional expression cassette further comprises at least one post-transcriptional regulatory element positioned upstream to one or each polyadenylation site. More preferably the bidirectional expression cassette further comprises at least one internal ribosome entry site (IRES) sequence to express three or more genes.

[0017] It is an object of the invention an expression construct containing the bidirectional promoter, as above disclosed.

[0018] It is an object of the invention an expression construct containing the bidirectional expression cassette, as above disclosed.

[0019] It is an object of the invention a gene transfer expression vector containing the expression construct as above disclosed further comprising lentiviral or retroviral sequences.

[0020] It is an object of the invention the use of the gene transfer expression vector for the preparation of a delivery and expression system in animal cells, preferably in vivo tissue animal cells, more preferably, brain neurons.

[0021] It is an object of the invention an in vitro method for the coordinate expression of two exogeneous coding sequences into an animal cell comprising the following steps:

a) cloning said coding sequences into the gene transfer expression vector according to claim 8, each coding sequence under the control of one of the two promoters of the bidirectional promoter;
b) transforming animal cells by means of said vectors;
c) allowing the expression of the vector.

[0022] Preferably the animal cell is an human cell, more preferably the human cell is a retransplantable human cell, even more preferably the retransplantable human cell is an hematopoietic cell.

[0023] Alternatively, the transformation of tissue cells in vivo may be performed by direct delivery of the vector, such as into brain neurons.

[0024] It is an object of the invention a method for generating a transgenic non human organism comprising the step of transforming appropriate cells by means of the gene transfer expression vector as disclosed above.

[0025] The vectors of the invention can be advantageously utilized for gene function and target validation studies in vitro and in vivo; gene therapy; expression of multiple genes in animal cells; generation of transgenic animals and eventually knock down of multiple genes; and for manufacturing of medicaments, as well.

FIGURE LEGENDS

[0026] The invention will be now described with reference to following Figures:

Fig. 1. Gene transfer performance of bicistronic lentiviral vectors. (a) Scheme of the proviral vector form. A bicistronic expression cassette containing an internal ribosome entry site (IRES) derived either from the encephalomyocarditis virus (EMCV), with wild-type (wt) or mutated (mut) translation start site, or from the 5' untranslated NF-kB repressing factor mRNA (NRF) was driven by the human immediate early cytomegalovirus (CMV) or phosphoglycerate kinase (PGK) promoter. ΔU3, R and U5, LTR regions with deletion in U3; SD and SA, splice donor and acceptor site; ψ, encapsidation signal including the 5' portion of the gag gene (GA); RRE, Rev-response element; cPPT, central polypurine tract; WPRE, woodchuck hepatitis virus post-transcription regulatory element. (b) Southern blot analysis of HeLa cells transduced by the indicated monocistronic (CMV) or bicistronic vectors expressing luciferase (gene 1) and GFP (gene 2) from the CMV promoter, probed for the WPRE sequence. All vector integrated with the expected length of DNA. Vector copy number was determined relative to a plasmid standard curve and used to normalize vector stocks and ensure similar levels of integration for each vector in a given target cell type in the experiments shown in c-f. (c-f) Luciferase and GFP expression in human HeLa cells (c), umbilical vein endothelial cells (HUVEC, d), peripheral blood lymphocytes (PBL, e), and cord blood-derived CD34+ progenitors (f) transduced 5-7 days before with a monocistronic (□, CMV) or the indicated bicistronic CMV-luciferase-GFP vector. Left column, histograms representing net luciferase activity in cells extracts, mean ± SD. Right panel, dot plots representing GFP expression by FACS analysis, the frequency and the mean fluorescence intensity (MFI, X) of GFP+ cells is indicated. The control monocistronic vector expressed luciferase in the histogram (□), and GFP in the leftmost dot plot (CMV) for each cell type. (g, h) FACS analysis of ΔNGFR and GFP expression in 293T cells (g) and CD34+ progenitors (h) transduced by a EMCV wt IRES vector expressing ΔNGFR and GFP from the PGK promoter. Histograms in panel (h) show the distribution of ΔNGFR expression in all viable cells analysed (left), and of GFP expression in the gated (M1) ΔNGFR+ cells (right). Experiments shown are representative of at least three performed with similar results.

Fig. 2. Gene transfer performance of bidirectional lentiviral vectors. (a) Scheme of the proviral vector form. A bidirectional promoter made by minimal core promoter elements from the human cytomegalovirus (mCMV) joined upstream, and in opposite orientation, to an efficient promoter, derived from the human phosphoglycerate kinase (PGK) or poly-ubiquitin UBI-C gene, was driving divergent transcription of two RNAs. CTE, constitutive transport element from the Mason-Pfizer monkey virus; pA, polyadenylation site A from the Simian Virus 40. Other vector features as in the legend to figure 1. (b) Net luciferase activity and (c-e) GFP expression in HeLa cells transduced 5-7 days before with LVs carrying the indicated bi-directional or control expression cassettes. The frequency and MFI (X) of GFP+ cells at FACS analysis is indicated in the dot plots to the right. Luciferase activity was determined for the two marked vectors (□, ■). (f-j) ΔNGFR and GFP expression in HeLa cells transduced 5-7 days before with serial 10-fold dilutions of LVs carrying the indicated expression cassette. The frequency of ΔNGFR+ (upper left region) and ΔNGFR/GFP double positive (upper right region) cells, with the respective MFI of ΔNGFR (Y) and GFP (X), are indicated in the FACS dot plots. Experiments shown are representative of at least three performed with similar results.

Fig. 3. Comparison of bi-directional and bicistronic lentiviral vectors performance. ΔNGFR and GFP expression in 293T cells transduced 3 weeks before with serial 10-fold dilutions of LVs carrying the indicated expression cassette. The total percentage of ΔLNGFR-expressing cells and of ΔLNGFR/GFP double positive cells (in brackets) are indicated above the FACS dot plots. The average number of vector Copies per Cell (CpC) is indicated in each plot, with the expected frequency of transduced cells according to the Poisson's distribution of random independent events. Although virtually all integrated vectors expressed ΔNGFR, its level of expression and the fraction of transduced cells co-expressing GFP were much higher for the two bi-directional vectors tested (MA1 and MA4) as compared to the EMCV wt IRES bicistronic vector.

Fig. 4. Dual-gene transfer in hematopoietic cells by bi-directional vectors. (a-c) Human cord blood CD34+ progenitors were transduced by the GFP-ΔNGFR MA1 vector in the presence of early acting cytokines as described[23], and analysed either after 7 days of culture in the same medium (a), and after additional 10 days in medium promoting myeloid differentiation (b), or after seeding in methylcellulose-based clonogenic medium. For (a) and (b), a dot plot showing ΔNGFR and GFP expression by FACS analysis is shown, together with histograms showing the distribution of ΔNGFR expression in all viable cells analysed (top), and of GFP expression in the gated (M1) ΔNGFR+ cells (bottom). The percentage of immature progenitors expressing CD34, and of differentiating cells expressing the CD 13 myeloid marker at the time of analysis is indicated. For (c), representative light (left) and fluorescent (right)

micrograph of the indicated type of CFC are shown. (d, e) Human peripheral blood lymphocytes were transduced either after 2-day activation with anti-CD3 and anti-CD28 antibodies (d), or after 4-day treatment with Interleukin-7, as described[24], (e), and analyzed for ΔNGFR and GFP expression as described above. (f, g) Purified (lin-) murine bone marrow progenitors were transduced without cytokine stimulation as described[48], and analyzed for ΔNGFR and GFP expression after 7 days in liquid culture (f), or immediately transplanted into lethally-irradiated syngenic recipients. FACS analysis of the peripheral blood of a representative mouse 2 months after transplant is shown in g. Experiments shown are representative of three performed with similar results. In d-f, cells transduced to low vector copy numbers are shown for more stringent performance analysis.

Fig. 5 In vivo dual-gene transfer by bi-directional vectors. High-titer of GFP-ΔNGFR MA1 LV were stereotactically injected into the striatum of adult mice. Cryostatic brain sections were obtained two months after injection and analyzed by immunofluorescence and confocal microscopy. Representative pictures of the injected area are shown, after immunostaining for ΔNGFR (red), GFP (green), and TO-PR03 staining for nuclear DNA (blue). Fluorescent signals were sequentially acquired from single optical sections and are shown individually and after merging (merge). Original magnification 200X (Scale bar =120 μm)

Fig. 6 Dual-transgenesis by bi-directional vector. Transgenic mouse lines were generated by direct injection of GFP-ΔNGFR MA1 LV into the perivitelline space of single-cell embryos, as described[19], and the indicated tissues were analyzed for ΔNGFR (red) and GFP (green) expression by immunofluorescence and confocal microscopy on cryostatic sections. Nuclei were stained by TO-PRO3 (blue). Fluorescent signals were sequentially acquired from single optical sections and are shown individually and after merging (merge). The pictures shown were obtained from an F1 mouse carrying two vector genomes integrated into the germ-line. Similar pictures were obtained from other transgenic mice analyzed that carried similar or higher number of vector copies. Original magnification 200X (spleen, lung), 400X (hearth, kidney, brain, liver), 630X (gut) (Scale bar = 120 μm)

Fig. 7a Map of the plasmid containing the lentiviral vector construct RRL-MA1-lucif/GFP.

Fig. 7b Sequence of the plasmid containing the lentiviral vector construct RRL-MA1-lucif/GFP.

Fig. 8a Map of the plasmid containing the lentiviral vector construct CCL-MA1-GFP/deltaLNGFR.

Fig. 8b Sequence of the plasmid containing the lentiviral vector construct CCL-MA1-GFP/deltaLNGFR.

Fig. 9a Map of the plasmid containing the lentiviral vector construct RRL-MA2-lucif/GFP.

Fig. 9b Sequence of the plasmid containing the lentiviral vector construct RRL-MA2-lucif/GFP.

Fig. 10a Map of the plasmid containing the lentiviral vector construct CCL-MA3-GFP/deltaLNGFR.

Fig. 10b Sequence of the plasmid containing the lentiviral vector construct CCL-MA3-GFP/deltaLNGFR.

Fig. 11a Map of the plasmid containing the lentiviral vector construct CCL-MA4-GFP/deltaLNGFR.

Fig. 11b Sequence of the plasmid containing the lentiviral vector construct CCL-MA4-GFP/deltaLNGFR.

EXAMPLE 1

MATERIALS AND METHODS

Plasmid construction

[0027] All transfer vectors were built from plasmid pCCL.sin.cPPT.PGK.GFP.WPRE [15] using the following previously described sequence elements: EMCV IRES's with the downstream gene coding sequence starting at the 11th ATG of the IRES (wt) or with the 11th ATG of IRES mutated to create a HindIII cloning site and allow translation initiation at the downstream transgene ATG [16](EMCVmut), the NRF IRES [18], the MPMV CTE[21], a minimal CMV core promoted[20], a 1226 bp fragment from the Ubiquitin-C promoter [19].

Construction of lentiviral vector with bi-directional promoters

[0028] To generate the lentiviral construct **RRL-MA1**, an XhoI-XhoI fragment containing the SV40polyA.CTE.Luciferase.minhCMV elements (derived from the lentiviral construct

[0029] pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minhCMV.TetO7.minMMTV.eGFP) was cloned into the lentiviral vector construct pRRL.sin.cPPT.hPGK.eGFP.Wpre (Follenzi et al., 2000) cut with the same enzyme to obtain RRL-MA1-lucif/GFP (pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minhCMV.hPGK.eGFP.Wpre).

[0030] To generate the lentiviral construct **CCL-MA1**, two fragments were cloned into the lentiviral construct pRRL.sin.cPPT.hPGK.ΔLNGFRWpre first cut with KpnI, blunted and then cut with XhoI, the first fragment containing the minhCMV.eGFP elements was derived from the lentiviral construct pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minMMTV.TetO7.minhCMV.eGFP cut with **KpnI**, blunted and then with XhoI and the second derived from the construct pRRL.sin.cPPT.SV40polyA.CTE.tTA2.Wpre cut with BamHI, blunted and then cut with NotI. The resulting lentiviral construct pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.minMMTV.TetO7.minhCMV.eGFP was cut with NotI and AvrII

and the fragment containing the cPPT.SV40polyA.CTE.eGFP.minhCMV.hPGK. ΔLNGFRWpre was cloned into the lentiviral construct pCCL.sin.cPPT.hPGK.eGFP.Wpre cut with the same enzymes to obtain CCL-MA1-GFP/ΔLNGFR (pCCL.sin.cPPT.SV40polyA.CTE.eGFP.minhCMV.hPGK. ΔLNGFRWpre).

[0031] To generate the lentiviral construct **RRL-MA2**, a HindIII-BamHI fragment containing the hFGK.Luciferase elements (derived from the lentiviral vector construct pRRL.sin.cPPT.hPGK.Luciferase.IRES.Wpre) was cloned into the retroviral construct SF2-cLCM2G (obtained from Rainer Loew, University of Heidelberg, FRG) cut with the same enzymes to obtain the construct cPPT.SV40polyA.CTE.Luciferase.hPGK.minMIVITV.eGFP. This construct was first cut with SalI, blunted and then cut with BamHI and the fragment containing the Luciferase.hPGK.minMMTV.eGFP elements was cloned into the lentiviral vector construct pRRL.sin.cPPT.SV40polyA.CTE.tTA2.Wpre cut in the same way, to obtain RRL-MA2-lucif/GFP (pRRL.sin.cPPT.SV40polyA.CTE.Luciferase.hPGK.minMMTV.eGFP.Wpre).

[0032] To generate the lentiviral construct **CCL-MA3**, two fragments were cloned into the pBLKS+ cut with HindIII and XhoI, the first fragment containing the CTE.SV40polyA elements was derived from the lentiviral vector construct pRRL.sin.cPPT.SV40polyA.CTE.tTA2 cut with HindIII and XbaI and the second fragment containing the minMMTV.GFP elements derived from the construct cPPT.SV40polyA.CTE.Luciferase.hPGK.minMMTV.eGFP cut with XhoI and XbaI to obtain the construct pBLKS+ minMMTV.GFP.CTE.SV40polyA. The resulting construct was cut with EcoRV and XhoI and the fragment containing the minMMTV.GFP.CTE.SV40polyA was cloned into the lentiviral vector construct pCCL.sin.cPPT.hPGK. ΔNGFR. Wpre cut with the same enzymes,to obtain the final lentiviral vector construct CCL-MA3-GFP/ΔNGFR (pCCL.sin.cPPT.SV40polyA.CTE.GFP.minMMTV.hPGK. ΔNGFR.Wpre)

[0033] To generate the lentiviral construct **CCL-MA4** the fragment derived from pHR'.UBI-C.eGFP cut with PacI, blunted and cut with PstI, containing the UBI-C promoter sequence, was inserted into the place of the PGK promoter into construct pCCL.sin.cPPT.SV40polyA.CTE.GFP.minCMV.PGKΔNGFR.Wpre cut with EcoRV and PstI to obtain the final lentiviral vector construct CCL-MA4-GFP/ΔNGFR (pCCL.sin.cPPT.SV40polyA.CTE.GFP.minCMV.UBI-C. ΔNGFR.Wpre)

[0034] The maps and the nucleotide sequences of the RRL-MA1-lucif/GFP, CCL-MA1-GFP/ΔLNGFR, RRL-MA2-lucif/GFF; CCL-MA3-GFP/ΔLNGFR; CCL-MA4-GFP/ΔLNGFR constructs are shown respectively in figures 7a-11a and figures 7b-11b.

Vector production and titration

[0035] VSV-pseudotyped third-generation LV were produced by transient 4-plasmid co-transfection into 293T cells and purified by ultracentrifugation as described [15], with the modification that **1 mM NaButyrate** was added to the cultures for vector collection[47]. Expression titer of GFP or ΔLNGFR vectors were estimated on HeLa cells by limiting dilution. Vector particle was measured by HIV-1 gag p24 antigen immunocapture (NEN Life Science Products). Vector infectivity was calculated as the ratio between titer and particle for the vector expressing GFP or ΔNGFR. Vector expression titer in the 293T supernatant ranged from 0.7 to 1x10^7 Transducing Units^{HeLa}(TU)/ml for monocistronic CMV or PGK vector, from 3 to 8x10^6 TU/ml for bicistronic vectors and bi-directional vectors. Vector infectivity ranged from 0.5 to 1x10^5 TU/ng of p24 for monocistronic CMV or PGK vector, and from 2 to 6x10^4 TU/ng of p24 for bicistronic and bi-directional vectors.

Cell cultures

[0036] Continuous cultures of HeLa and 293T cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Sigma, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen Corporation, UK) and a combination of penicillin-streptomycin and glutamine. Primary cultures of human umbilical vein endothelial cells (HCTVECs), peripheral blood lymphocytes, and cord blood CD34+ progenitors were obtained and maintained as described [15]. CD34+ progenitors were transduced with 5x10^7 TU/ml of LV and cultured for at least 7 days in the presence of recombinant human interleukin 6 (rhIL6, 20 ng/ml), recombinant human stem cell factor (rhSCF, 100 ng/ml), recombinant human FLT-3 ligand (rhFLT-3 ligand, 100ng/ml), all from PeproTech (Rocky Hill, NJ), and recombinant human thrombopoietin (rhTPO, 20 ng/ml; Amgen, Thousand Oaks, CA) as described[23]. For differentiating conditions, transduced progenitors were cultured for 10 days in the presence of rhSCF, 50 ng/ml, recombinant human granulocyte monocyte-colony stimulating factor (rhGM-CSF, 20 ng/ml), recombinant human monocyte-colony stimulating factor (rhG-CSF, 20 ng/ml), all from PeproTech. For clonogenic assays, transduced cells were plated at a density of 800 cells/ml in human complete MethoCult medium (StemCell Technologies, Vancouver, CA) and were scored by light and fluorescence microscopy 14 days later.

[0037] Human peripheral blood lymphocytes were purified by Ficoll gradient and transduced with 0.5-5x10^7 TU/ml of vector either after 2-day activation with 30ng/ml anti-CD3 antibodies (Orthoclone, Milan, Italy) plus 1μg/ml anti-CD28 antibodies (PharMingen, San Diego, CA), or after 4-day treatment with 5ng/ml interleukin-7 (Boehringer Mannheim-Roche GmbH, Mannheim, Germany), as described[24].

[0038] Purification of lineage marker-negative cells from C57BL/6 mouse bone marrow with a magnetic cell depletion

technique (StemCell Technologies, Vancouver, CA), *ex vivo* transduction in serum-free StemSpan medium (StemCell Technologies, Vancouver, CA) with 0.5-2x10^7 TU/ml of vector, and transplantation into lethally irradiated syngenic recipients were performed as described [48].

Mice

[0039] CD1, C57BL/6 and FVB mice were purchased from Charles Rivers Laboratories (Calco, Italy) and maintained in SPF conditions. All animal procedures were performed according to protocols approved by the Hospital San Raffaele Institutional

[0040] Animal Care and Use Committee.

DNA analysis: Southern and real time PCR

[0041] Vector copies per genome were quantified by Real-Time PCR from 300 ng template DNA extracted from cells by a commercial kit (Qiagen), using one set of primers and probe to detect the LV backbone:

LV forward primer, 5'-TGAAAGCGAAAGGGAAACCA-3';
LV reverse primer, 5'-CCGTGCGCGCTTCAG-3';
LV probe, 5'-(VIC)-CTCTCTCGACGCAGGACT-(TAMRA)-3'.

[0042] Reactions were carried out according to manufacturer instructions and analysed using the ABI Prism 7700 sequence detection system (PE-Applied Biosystem). For Southern blot, DNA was extracted from transduced cells, digested with AfiI to release the expression cassette from integrated vector DNA and analysed with a WPRE probe to detect vector sequences. The average number of integrated vector copies was determined relative to a plasmid standard curve.

[0043] These numbers were used to calculate vector integration titer and normalize vector stocks for all subsequent transduction experiments to ensure similar levels of integration for each vector tested.

Experimental Design and Stereotactic Injection.

[0044] Nine weeks-old C57BL/6 mice were anesthetized with intraperitoneal injection of Tribromoethanol 1.25% (SIGMA), positioned in a stereotactic frame (David Kopf Instruments, Tujunga, CA) and the skull exposed by a small incision. Two µl of vector concentrate (2 x10^6 TU/µl) was injected by a Hamilton syringe with a 33G blunt tip needle (Hamilton; Reno, NV) into the left hemisphere striatum (stereotactic coordinates in mm from bregma: AP=+0.74, ML=-1.9 and DV=-3.5 from skull surface) at a rate of 0.2 µl/min. The needle was left in place for additional 5 minutes before slow removal.

Transgenesis

[0045] Transgenic mice were generated using LV as described by Lois et al.[19]. Briefly, female FVB mice were superovulated with a combination of pregnant mare serum and human chorionic gonadotropin. On average between 20 and 30 embryos were collected per female and microinjected into the perivitelline space with 10-100 pL of 5x10^7 TU/ml LV stock on the same day. Manipulated embryos were immediately implanted into the oviduct of pseudopregnant CD1 mice. Pups were genotyped for the presence of the GFP sequence by PCR analysis as described[49]. Positive mice were bred to test germ-line trasmission of the transgene. DNA was extracted from the tail and used to quantify vector copy number by real time PCR in founder and F1 progeny mice.

Flow cytometry and Luciferase assay

[0046] Transduced cells were grown for at least 4 days before FACS analysis to reach steady state GFP expression and to rule out pseudotransduction. Before FACS analysis, adherent cells were detached with 0.05% trypsin-EDTA, washed, and fixed in phosphate buffer saline (PBS) containing 1% paraformaldehyde (PAF) and 2% FBS. Cells grown in suspension were washed and resuspended in PBS containing 2 µg/ml propidium iodide (PI) (BD Bioscience PharMingen, San Diego, CA) and 2% FBS. For immunostaining, 10^5 cells were blocked in PBS 5% mouse serum, 5% human serum, 2% FBS for 15 min at 4°C. After blocking, 10µl of R-phycoerythrin (RPE)-conjugated antibodies (anti-CD34 and anti-CD13, Dako, Glostrup, Denmark, and anti-ΔLNGFR, BD Bioscience PharMingen, San Diego, CA) were added and the cells were incubated for 30 min at 4°C, washed, stained with PI, and analyzed by three-color flow cytometry. Only viable, PI-negative cells were used for the analysis.

[0047] Luciferase was assayed in cell lysates prepared as described by the manufacturer (luciferase assay system,

Promega). RLU were measured with a Lumat LB9507 luminometer (Berthold) after mixing cell lysates (normalized for protein content measured by BCA Protein Assay Reagent kit Pierce) with Luciferase Substrate (Promega).

Tissue analysis

[0048] Anesthetized mice were perfused with 0.9% NaCl followed by 4% PAF in PBS. Tissue samples were collected, equilibrated in 20% sucrose in PBS for 48 h at 4°C, and embedded in optimal-cutting-temperature compound (OCT) for quick freezing. 10µm (for transgenic mice) and 20µm (for stereotactic injected mice) thick cryostatic sections were post-fixed in PAF and frozen at -80 °C. Sections were blocked with 5% goat serum (Vector Laboratories) in PBS containing 1% bovine serum albumine (BSA) and 0.1% Triton X-100 (PBS-T), and incubated with rabbit affinity-purified GFP antibody (Molecular Probes) and R-phycoerythrin (RPE)-conjugated ΔLNGFR monoclonal antibody (BD Bioscience PharMingen, San Diego, CA) for 1 h, washed and stained with AlexaFluor488-conjugated goat anti-rabbit antibody (Molecular Probes) in PBS-T and 1% BSA for 1 h. Cell nuclei were stained with TOPRO-3 after 1h of RNAse treatment (Molecular Probes). Sections were mounted and analyzed by three-laser confocal microscope (Radiance 2100; BioRad). Fluorescent signals from single optical sections were sequentially acquired and analyzed by PhotoShop 7.0 (Adobe).

RESULTS

Bicistronic LVs

[0049] In order to express more than one transgene from a single vector, the authors first evaluated the performance of different IRES's in the context of late-generation self-inactivating LVs[15]. They used the strong CMV and PGK promoters to drive expression of bicistronic transcripts encoding, from the 5' to the 3' end, the luciferase reporter, an IRES, and the cell-associated GFP marker (fig. 1a). Two IRES's were derived from the Encephalomyocarditis virus; a wild-type (EMCVwt) and a mutant (EMCVmut) form [16, 17], that differed for the ATG from which downstream translation started. Another IRES was derived from the 5' untranslated sequence of the NF-kB transcription Repressing Factor (NRF) mRNA[18].

[0050] They generated high-titer VSV-pseudotyped stocks of all bicistronic and control monocistronic vectors, and normalized them for transducing activity measuring integration in HeLa cells by Southern blot (fig. 1b). They then compared gene expression in cells transduced to equal vector copy numbers (Fig. 1c-f). Although luciferase activity was similar in HeLa cells transduced by CMV-luciferase vector and in cells transduced by the best performing bicistronic vector, only a small fraction of the latter cells expressed the IRES-dependent GFP gene, with a ten-fold decrease in expression titer as compared to cells transduced by the control CMV-GFP vector (Fig. 1c). Moreover, the GFP mean fluorescence intensity (MFI) was significantly lower in cells expressing the protein from the IRES's than in cells expressing it from the ^{mRNA}Cap. They then tested bicistronic LVs in primary human cells, including umbilical vein endothelial cells, peripheral blood lymphocytes, and CD34+ cord blood hematopoietic progenitors (HPC) (Fig. 1d-f). All cell types were transduced efficiently, as indicated by the frequency of GFP-positive cells in cultures transduced by control CMV-GFP vector, but IRES-dependent GFP expression was only observed in a fraction of cells transduced by bicistronic vectors. IRES activity varied extensively with the target cell type; the NRF IRES was the only one reaching detectable downstream gene expression in lymphocytes, while the EMCVwt IRES was the most efficient in the other cell types. In addition, all IRES's decreased, in some cases more than one log, upstream gene expression, as compared to the control CMV-luciferase vector.

[0051] They also evaluated IRES-based vectors by expressing two cell-associated markers, GFP and a truncated version of the low-affinity NGF receptor (ΔLNGFR) (Fig. 1g,h). Among HeLa cells transduced by a low dose of the best-performing bicistronic vector, only the cells expressing high levels of ΔLNGFR also expressed GFP, with an average of one out of four ΔNGFR-positive cells expressing GFP to detectable levels (Fig. 1g). Similarly, only a small fraction of transduced CD34+ progenitors expressing ΔNGFR also expressed GFP to detectable levels (Fig. 1h). Overall, these results indicated that IRES-based bicistronic vectors failed to ensure coordinate expression of two transgenes in most target cell types tested, and that multi-copy transduction or selection of transduced cells for downstream gene expression were required to obtain a population expressing both transgenes in the majority of cells. *Bidirectional LVs*

[0052] To overcome the limitations of bicistronic vectors, the authors explored a new promoter design for coordinate transgene expression. They joined a minimal core promoter upstream, and in opposite orientation, to an efficient promoter. Rationale of this design was that upstream elements in the efficient promoter, when closely flanked by core promoters on both sides, may drive transcriptional activity in both directions. If such bi-directional activation occurred, expression of both transcripts would be coordinately regulated. They tested two ubiquitously expressed promoters, previously shown to drive robust and efficient transgene expression in LV; the above mentioned 516 bp fragment from the human phosphoglycerate kinase promoter (PGK) [15] and a 1226 bp fragment from the human ubiquitin C promoter (UBI C)[19]. They joined them to a minimal core promoter derived from the cytomegalovirus (minCMV) that was previously developed to

couple initiation of eukaryotic transcription to tetracycline (Tc)-dependent operators[20]. They flanked the bi-directional promoter with two expression cassettes optimized for LV-mediated gene delivery (fig. 2a). The upstream cassette - in anti-sense orientation relative to the vector LTR - included the constitutive transport element (CTE) of the Mason-Pfizer virus[21], and a polyadenylation site from the Simian Virus 40 (SV40). The downstream cassette included the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE)[22] and the SIN HIV-1 LTR polyadenylation site.

[0053] As described above for bicistronic LVs, they verified correct transfer and normalized transduction of each vector by Southern blot analysis and real-time PCR of transduced cells. LV carrying bi-directional expression cassettes were produced to high titer and infectivity, similar to those obtained with standard vectors (see Methods). The bi-directional design significantly enhanced transcription from the upstream minimal promoter without affecting downstream expression from the efficient promoter (fig. 2b-h). Luciferase expression from the minCMV promoter, for instance, was increased at least one log when fused upstream to the PGK promoter (fig. 2b). Remarkably, the bi-directional PGK promoter allowed detecting GFP (or ΔLNGFR, not shown) to the same frequency and to similar expression levels in cells transduced by the bi-directional vector and expressing the protein from either side of the promoter (fig. 2c,d), as in cells transduced by the control PGK vector (fig. 2e). Using two cell-associated markers, ΔLNGFR and GFP, they showed stable, efficient and coordinate expression of bi-directional LVs, both at high and low vector copy number (fig. 2f). At high vector input, they reached high-level expression of both transgenes in virtually every target cell. At low vector input, when most transduced cells carried one proviral copy, they showed transgene co-expression in virtually every labeled cell, indicating the occurrence of divergent transcription from the bi-directional promoter. In both conditions, transgene expression was maintained to similar levels in cells analyzed at early and late times post-transduction (not shown, and Fig. 3 below). Transgene-expressing cells tended to distribute along a diagonal line in the two-color FACS plot, indicating that expression of the two transgenes was coordinately regulated.

[0054] Intriguingly, they observed coordinate bi-directional expression, although to significantly lower efficiency on the upstream side than the downstream side, when they tested the sole PGK promoter in the context of the bi-directional expression cassette that they developed (fig. 2g). They reproduced this finding after swapping the position of the two transgenes on each sides of the PGK promoter (not shown). These results indicated that transcription-activating elements in the PGK promoter are intrinsically capable of triggering divergent transcription and thus provide the main driving force for dual-gene expression in the new LV, ensuring coordinate regulation of transcription on both sides of the bi-directional promoter. Apposition of the minCMV core promoter, which had a very low activity per se (fig. 2h, and 2b above), enhanced upstream transcription from the PGK promoter possibly because of more efficient initiation (compare fig. 2g and 2f). When they changed the driving promoter in bi-directional vectors from PGK to UBI-C, they reproduced the findings observed with the PGK promoter (fig. 2i). They revealed an intrinsic bi-directional activity of the UBI-C promoter (fig. 2j) that was significantly enhanced by the upstream addition of the minCMV promoter.

[0055] They then compared directly the performance of bi-directional and bicistronic vectors in relation to the number of integrated copies, as measured by real-time PCR (Fig. 3). By analyzing 293T cells transduced with increasing vector doses, they proved that the vast majority of integrated bi-directional vectors based on the PGK (MA1) or UBI-C (MA4) promoter efficiently expressed both transgenes, clearly outperforming the best IRES-based bicistronic vector.

Ex Vivo and In Vivo Dual-Gene Transfer

[0056] They then assessed the performance of the bi-directional MA1 LV in more relevant targets for gene therapy applications and by different delivery strategies. They transduced human cord-blood HPC and PBL with ΔLNGFR-GFP MA1 LV *ex vivo*, according to previously optimized protocols[23, 24] (Fig.4). Both gene products were coordinately expressed to high-levels in a large fraction of HPC scored both as immature cells grown in the presence of early-acting cytokines (Fig. 4a), and after differentiation in liquid culture (Fig. 4b) or clonogenic assay (Fig. 4c, GFP only). Similarly, they obtained coordinate ΔLNGFR and GFP expression in PBL transduced in standard conditions of proliferation, triggered by CD3/CD28 co-stimulation (Fig. 4d), and as non-proliferating cells, treated only with IL-7 to maintain naive cell properties (Fig. 4e). They also performed transplantation studies with transduced murine HPC, enriched from the bone marrow by negative selection, to prove stable dual-transgene expression in the progeny of long-term repopulating HSC (Fig 4f). ΔLNGFR and GFP were coordinately expressed to similar levels in the *ex vivo* transduced cells, before transplantation, and in the white blood cells of long-term engrafted mice. Overall, these results validated the new LV for proficient dual gene transfer in primitive, committed, and differentiated hematopoietic cells.

[0057] They injected concentrated ΔLNGFR-GFP MA1 LV in the striatum of adult mice and scored transgene expression 4 weeks after injection by confocal microscopy of brain sections immuno-stained for GFP and ΔLNGFR (fig. 5). They observed robust co-expression of both transgenes in the brain tissue surrounding the injection site. As previously reported after striatal injection of VSV-pseudotyped LV[25-27], the vast majority of cells expressing the markers had the typical morphology of striatal neurons. Thus, the new bi-directional LV enabled efficient *in vivo* dual-gene transfer.

Dual-Transgenesis

[0058] They evaluated whether the new bi-directional LV allowed generation of dual-transgenic mouse lines. As previously described by Lois et al[19], they microinjected the ΔNGFR-GFP LV into the perivitelline space of single-cell embryos, and implanted them into pseudopregnant females. We obtained transgenic mice to high frequency, as assessed by the presence of vector DNA (more than 50% of newborns), and proved vector integration in the germ line by crossing some founder mice and analyzing their progeny for vector DNA content and transgene expression (Fig. 6). In the two F1 mice analyzed, carrying 2 and 5 vector copies in the genome, they found remarkably consistent expression of both transgenes in virtually every cell in the tissues studied, which included brain, liver, spleen, gut, heart, skeletal muscle, and kidney. Vector expression was also well detectable in the bone marrow and peripheral blood of the same mice, although in less than 100% of the cells, and more clearly for ΔNGFR than GFP (not shown). These data indicated that bi-directional LV transgenesis is a rapid and efficient method to obtain robust, stable and coordinate expression of two transgenes in genetically-engineered mice. In addition, they show that the minCMV-PGK bi-directional promoter that they developed governs dual transgene expression in the majority of differentiated tissues of the mouse, and maintains expression after inheritance through the germ-line.

DISCUSSION

[0059] In the pursuit of strategies enabling efficient dual-gene transfer, they initially faced significant limitations of IRES-based approaches. When tested in the context of bicistronic LV, IRES-dependent gene expression was significantly lower than that dependent on the ^{mRNA}Cap, and required multi-copy transduction to co-express the downstream gene in a sizable fraction of transduced cells. In addition, IRES's decreased expression of the upstream gene in the transcript, and displayed significant cell type-dependent variation in activity. Similar limitations have been reported when incorporating IRES's into other types of gene transfer vectors[14,28-32]. Thus, selection for downstream gene expression is likely to be required when using IRES to ensure co-expression in all target cells. Although selection protocols are compatible with some *ex vivo* gene transfer and therapy applications, they may adversely affect the biological properties of gene-corrected cells, in particular when selectable marker expression is inefficient. In fact, prolonged *ex vivo* culture and a limited size or clonal composition of the transduced cell population may reduce engraftment, long-term survival and tissue repopulation after transplantation[33]. Even more important, the inefficiency of IRES-dependent expression prevents most application of bicistronic vectors to direct *in vivo* gene transfer. Thus, authors explored novel strategies to take full advantage of gene transfer systems, such as LV, that allow efficient ex vivo transduction and direct *in vivo* administration[34].

[0060] They have developed a new promoter design based on the juxtaposition of core promoter elements upstream, and in opposite orientation, to an efficient promoter. The bi-directional assembly drove divergent transcription, indicating that upstream enhancer/promoter elements within the efficient promoter were capable of promoting transcription in an orientation-independent manner and from both sides simultaneously. Upon incorporation of these promoters into LV, they reached efficient dual-gene transfer and coordinate expression in continuous cell lines and primary cells *ex vivo*. Because both transgenes were expressed in the vast majority of transduced cells, they did not need to select cells to ensure transgene co-expression. Upon direct injection of bi-directional LV into the CNS, the authors showed coordinate expression of two transgenes in neural cells *in vivo*. In addition, bi-directional LV allowed robust dual transgenesis, leading to pan-cellular expression of both transgenes in all tissues examined. All these results could not be reached until now using currently available technologies.

[0061] By monitoring transduced cells carrying a single vector copy, authors proved that divergent transcription occurred from a single bi-directional promoter, that expression of both transgenes was functionally linked and coordinately regulated, and that bi-directional promoters were consistently active in all types of target cells tested, without being silenced or randomly fixed in one direction of transcription, even after cellular differentiation. Although they did not map how close the two opposite core promoters must be for operational linkage, they may expect that close juxtaposition of the fused minimal core promoter to some of the upstream elements in the efficient promoter, as observed in natural promoters between core and upstream elements, may be required. Both the PGK and UBI-C promoters tested in this work drove divergent transcription when fused to a minimal core promoter in the opposite orientation. Intriguingly, both of these promoters were shown to be intrinsically capable of promoting divergent transcription, although to lower efficiency on the upstream than the downstream side, when incorporated into the bi-directional expression cassette that they developed. This surprising observation may indicate a specific feature of a class of ubiquitously-expressed housekeeping promoters, possibly related to their content of CpG islands (see below and[35-37]). However, they should not forget that both the promoter placement between two efficient expression cassettes endowed with post-transcriptional regulatory elements enhancing translation, and LV-mediated integration, which has been shown to preferentially target transcribed genes in the chromatin, may contribute to unravel latent transcriptional activity. Although the intrinsic bi-directional activity of the housekeeping promoters tested may not be efficient enough for exploitation *per se*, without the upstream assembly of core promoter elements described in this work, it provides the basis for the coordinate regulation of dual-gene ex-

pression reached by our new vectors. On the other hand, the propensity of these promoters to drive divergent transcription should be kept in mind when engineering vectors and analyzing transduced cells or tissues[38], and may provide a possible mechanism for the frequently observed interference between nearby promoters in the same vector construct [10,39]. It is possible that the bi-directional design described here may be successfully applied to tissue-specific promoters to obtain coordinated expression of two transgenes in specific tissues. In addition, by combining bi-directional promoters with bicistronic transcripts one could express more than two transgenes within the same cell, although with the limitations described above for IRES-dependent vectors.

[0062] Inducible bi-directional promoters were originally developed in Tet-regulated expression systems, by duplicating a minimal promoter on both sides of a series of Tet operator repeats, to obtain exogenously regulated expression of two transgenes[36, 40,41]. This design was recently applied to other systems that also combine prokaryotic enhancer elements with chimeric trans-activators to regulate gene expression[42]. Although these inducible expression systems represent powerful tools for gene-function studies, they are dependent on co-expression and functional activity of protein trans-activators, and pose several challenges when applied to vector-based delivery and *in vivo* applications. A constitutive bi-directional promoter was recently tested for exogenous gene expression in plant biotechnology[43]. Our results provide the first description of synthetic bi-directional promoters that exploit the endogenous transcriptional machinery available to most animal cell types to drive robust and constitutive expression of two divergent transcripts. In nature, few instances of bi-directional promoters had been documented until recently. Intriguingly, a recent survey of the human genome indicated an abundance of divergently transcribed gene pairs, whose transcription start sites are separated by less than 1 kb [44,45]. It is likely that many of the promoter elements found between these gene pairs can initiate transcription in both directions, and contain shared elements that regulate both genes[46]. Thus, the synthetic bi-directional promoters that they have developed may mimic a well-represented and evolutionary conserved feature of eukaryotic transcription, providing a structural basis for their robust performance. The new lentiviral vectors built around these bi-directional promoters will likely advance the reach and the safety of gene therapy, the power of gene-function and target validation studies, and the applications of animal transgenesis. If adapted for the expression of short interfering RNA, they may also enable coordinate knock-down of multiple genes.

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[0063]

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SEQUENCE LISTING

[0064]

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<120> LENTIVIRAL VECTORS CARRYING SYNTHETIC BI-DIRECTIONAL PROMOTERS AND USES THEREOF

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gaccttcctt cccgcggcct gctgccggct ctgcggcctc ttccgcgtct tcgccttcgc 8700
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ggggactgga agggctaatt cactcccaac gaagacaaga tctgcttttt gcttgtactg 8880
ggtctctctg gttagaccag atctgagcct gggagctctc tggctaacta gggaacccac 8940
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ttacaacgtc gtgactggga aaaccctggc gttacccaac ttaatcgcct tgcagcacat 9540
ccccctttcg ccagctggcg taatagcgaa gaggcccgca ccgatcgccc ttcccaacag 9600
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```
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acgttggagt ccacgttctt taatagtgga ctcttgttcc aaactggaac aacactcaac   9960
cctatctcgg tctattcttt tgatttataa gggattttgc cgatttcggc ctattggtta  10020
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atttcc                                                             10086
```

Claims

1. A bidirectional promoter for expression of at least two coding sequences in opposite direction in animal cells comprising 5' end to 3' end:

   a) a first minimal promoter sequence derived from cytomegalovirus (CMV) or mouse mammary tumor virus (MMTV) genomes;
   b) a promoter sequence derived from an animal gene comprising an enhancer region and a second minimal promoter sequence;

   the two promoter sequences driving a coordinate transcription of said coding sequences in the opposite orientation.

2. The bidirectional promoter according to claim 1 wherein the full efficient promoter sequence derives from ubiquitously expressed genes comprising the phosphoglycerate kinase or the ubiquitin gene.

3. A bidirectional expression cassette essentially comprising the bidirectional promoter according to previous claims, convenient insertion sites positioned downstream to each promoter, and polyadenylation sites positioned downstream to each insertion site.

4. The bidirectional expression cassette according to claim 4 further comprising at least one post-transcriptional regulatory element positioned upstream to one or each polyadenylation site.

5. The bidirectional expression cassette according to claim 4 or 5 further comprising at least one internal ribosome entry site (IRES) sequence to express three or more genes.

6. An expression construct containing the bidirectional promoter according to claim 1 or 2.

7. An expression construct containing the bidirectional expression cassette according to claims 4-6.

8. A gene transfer expression vector containing the expression construct according to claims 7 or 8 further comprising lentiviral or retroviral sequences.

9. Use of the gene transfer expression vector according to claim 8 for the preparation of a delivery and expression system in animal cells.

10. Use of the gene transfer expression vector according to claim 9 wherein animal cells are tissue animal cells in vivo.

11. Use of the gene transfer expression vector according to claim 10 wherein tissue animal celles are comprising brain neurons.

12. An in vitro method for the coordinate expression of two exogeneous coding sequences into an animal cell comprising the following steps:

    a) cloning said coding sequences into the gene transfer expression vector according to claim 8, each coding sequence under the control of one of the two promoters of the bidirectional promoter;
    b) transforming animal cells by means of said vectors;

c) allowing the expression of the vector.

13. The in vitro method for the coordinate expression of two exogeneous coding sequences according to claim 12 wherein the animal cell is an human cell.

14. The in vitro method for the coordinate expression of two exogeneous coding sequences according to claim 13 wherein the human cell is a retransplantable human cell.

15. The in vitro method for the coordinate expression of two exogeneous coding sequences according to claim 14 wherein the retransplantable human cell is an hematopoietic cell.

16. Method for generating a transgenic non human organism comprising the step of transforming appropriate cells with an expression construct containing the bidirectional cassette according to claims 6 or 7.

17. Method for generating a transgenic non human organism comprising the step of transforming appropriate cells by means of the gene transfer expression vector according to claim 8.

Patentansprüche

1. Bidirektionaler Promotor für die Expression von mindestens zwei Kodierungssequenzen in entgegen gesetzter Richtung in Tierzellen, umfassend vom 5'-Ende zum 3'-Ende:

   a) eine erste minimale Promotorsequenz, die aus dem Genom des Zytomegalievirus (ZMV) oder des Mausmammatumorvirus (MMTV) stammt;
   b) eine Promotorsequenz, die aus einem Tiergen stammt und die eine Verstärker (Enhancer)-Region und eine zweite minimale Promotorsequenz aufweist;

   wobei die beiden Promotorsequenzen eine koordinierte Transkription der Kodierungssequenzen in entgegen gesetzter Richtung steuern.

2. Bidirektionaler Promotor nach Anspruch 1, wobei die voll effiziente Promotorsequenz aus ubiquitär exprimierten Genen stammt, welche das Phosphoglyceratkinasegen oder das Ubiquitingen umfassen.

3. Bidirektionale Expressionskassette, die im Wesentlichen den bidirektionalen Promotor nach den vorherigen Ansprüchen, stromabwärts (downstream) von jedem Promotor positionierte, geeignete Insertionsstellen und (downstream) von jeder Insertionsstelle positionierte Polyadenylierungsstellen aufweist.

4. Bidirektionale Expressionskassette nach Anspruch 4, die des Weiteren mindestens ein stromaufwärts (upstream) von einer oder jeder Polyadenylierungsstelle positioniertes posttranskriptionales regulatorisches Element aufweist.

5. Bidirektionale Expressionskassette nach Anspruch 4 oder 5, die des Weiteren mindestens eine interne Ribosomeneintrittsstellen(IRES)-Sequenz aufweist, um mindestens drei Gene zu exprimieren.

6. Expressionskonstrukt, welches den bidirektionalen Promotor nach Anspruch 1 oder 2 enthält.

7. Expressionskonstrukt, welches die bidirektionale Expressionskassette nach Anspruch 4-6 enthält.

8. Gentransferexpressionsvektor, welcher das Expressionskonstrukt nach Anspruch 7 oder 8 enthält und des Weiteren lentivirale oder retrovirale Sequenzen aufweist.

9. Verwendung des Gentransferexpressionsvektors nach Anspruch 8 für die Herstellung eines Einführungs- und Expressionssystems in Tierzellen.

10. Verwendung des Gentransferexpressionsvektors nach Anspruch 9, wobei Tierzellen Tiergewebezellen in vivo sind.

11. Verwendung des Gentransferexpressionsvektors nach Anspruch 10, wobei Tiergewebezellen Gehirnneuronen umfassen.

12. In-vitro-Verfahren für die koordinierte Expression zweier exogener Kodierungssequenzen in eine Tierzelle, umfassend die folgenden Schritte:

    a) Klonieren der Kodierungssequenzen in den Gentransferexpressionsvektor nach Anspruch 8, wobei jede Kodierungssequenz unter der Kontrolle von einem der beiden Promotoren des bidirektionalen Promotors steht;
    b) Transformieren von Tierzellen mittels der Vektoren;
    c) Zulassen der Expression des Vektors.

13. In-vitro-Verfahren für die koordinierte Expression zweier exogener Kodierungssequenzen nach Anspruch 12, wobei die Tierzelle eine menschliche Zelle ist.

14. In-vitro-Verfahren für die koordinierte Expression zweier exogener Kodierungssequenzen nach Anspruch 13, wobei menschliche Zelle einer retransplantierbare menschliche Zelle ist.

15. In-vitro-Verfahren für die koordinierte Expression zweier exogener Kodierungssequenzen nach Anspruch 14, wobei die retransplantierbare menschliche Zelle eine hämatopoietische Zelle ist.

16. Verfahren zum Herstellen eines transgenen, nicht menschlichen Organismus, das den Schritt des Transformierens geeigneter Zellen mit einem Expressionskonstrukt, das die bidirektionale Kassette nach Anspruch 6 oder 7 enthält, umfasst.

17. Verfahren zum Herstellen eines transgenen, nicht menschlichen Organismus, das den Schritt des Transformierens geeigneter Zellen mittels des Gentransferexpressionsvektors nach Anspruch 8 umfasst.

Revendications

1. Promoteur bidirectionnel pour l'expression d'au moins deux séquences codantes dans une direction opposée dans des cellules animales comprenant, de l'extrémité 5' à l'extrémité 3' :

    a) une première séquence promotrice minimale dérivée des génomes du cytomégalovirus (CMV) ou du virus de la tumeur mammaire murine (MMTV) ;
    b) une séquence promotrice dérivée d'un gène animal comprenant une région amplificatrice et une seconde région promotrice minimale ;

    les deux séquences promotrices régissant une transcription coordonnée desdites séquences codantes dans l'orientation opposée.

2. Promoteur bidirectionnel selon la revendication 1, dans lequel la totalité de la séquence promotrice efficace dérive de gènes exprimés de manière ubiquitaire comprenant la phosphoglycérate kinase ou le gène de l'ubiquitine.

3. Cassette d'expression bidirectionnelle comprenant essentiellement le promoteur bidirectionnel selon les revendications précédentes, des sites d'insertion adaptés positionnés en aval de chaque promoteur, et des sites de polyadénylation en aval de chaque site d'insertion.

4. Cassette d'expression bidirectionnelle selon la revendication 4, comprenant en outre au moins un élément de régulation post-transcriptionnel positionné en amont de l'un des ou de chaque site(s) de polyadénylation.

5. Cassette d'expression bidirectionnelle selon la revendication 4 ou 5, comprenant en outre au moins une séquence IRES (site d'entrée interne du ribosome) pour exprimer trois gènes ou plus.

6. Construit d'expression contenant le promoteur bidirectionnel selon la revendication 1 ou 2.

7. Construit d'expression contenant la cassette d'expression bidirectionnelle selon les revendications 4 à 6.

8. Vecteur d'expression de transfert de gènes contenant le construit d'expression selon la revendication 7 ou 8, comprenant en outre des séquences lentivirales ou rétrovirales.

9. Utilisation d'un vecteur d'expression de transfert de gènes selon la revendication 8, pour la préparation d'un système de délivrance et d'expression dans les cellules animales.

10. Utilisation du vecteur d'expression de transfert de gènes selon la revendication 9, dans laquelle les cellules animales sont des cellules de tissu animal in vivo.

11. Utilisation d'un vecteur d'expression de transfert de gènes selon la revendication 10, dans laquelle les cellules de tissu animal comprennent des neurones cérébraux.

12. Procédé *in vitro* pour l'expression coordonnée de deux séquences codantes exogènes dans une cellule animale comprenant les étapes suivantes :

a) le clonage desdites séquences codantes dans le vecteur d'expression de transfert de gènes selon la revendication 8, chaque séquence codante sous le contrôle de l'un des deux promoteurs du promoteur bidirectionnel ;
b) la transformation de cellules animales par les moyens desdits vecteurs ;
c) le fait de permettre l'expression du vecteur.

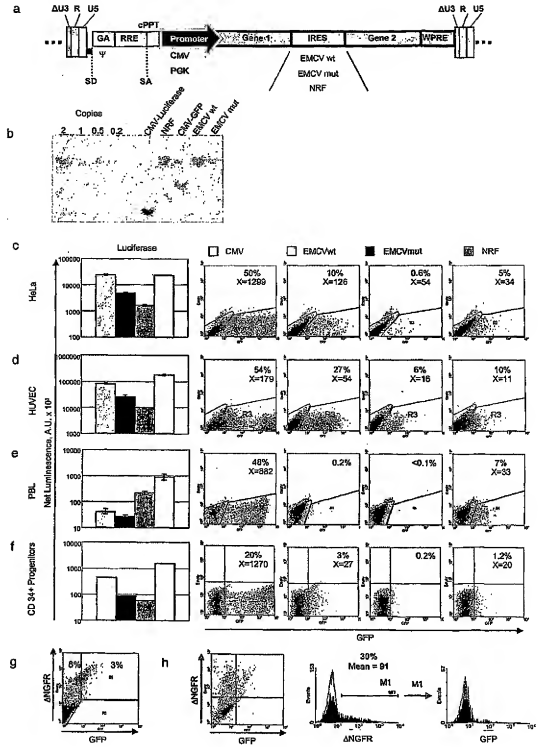
13. Procédé *in vitro* pour l'expression coordonnée de deux séquences codantes exogènes selon la revendication 12, dans lequel la cellule animale est une cellule humaine.

14. Procédé in vitro pour l'expression coordonnée de deux séquences codantes exogènes selon la revendication 13, dans lequel la cellule humaine est une cellule pouvant être retransplantée.

15. Procédé in vitro pour l'expression coordonnée de deux séquences codantes exogènes selon la revendication 14, dans lequel la cellule humaine pouvant être retransplantée est une cellule hématopoïétique.

16. Procédé de génération d'un organisme transgénique non humain comprenant l'étape consistant à transformer des cellules adaptées avec un construit d'expression contenant la cassette bidirectionnelle selon les revendications 6 ou 7.

17. Procédé de génération d'un organisme transgénique non humain comprenant l'étape consistant à transformer des cellules adaptées par les moyens du vecteur d'expression de transfert de gènes selon la revendication 8.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ΔU3 R U5
cPPT
GA
RRE
Ψ
SD
SA
Promoter
CMV
PGK
Gene 1
IRES
EMCV wt
EMCV mut
NRF
Gene 2
WPRE
ΔU3 R U5
b
Copies
2 1 0.5 0.2
CMV-Luciferase
NRF
CMV-ΔNGFR
EMCV wt
EMCV mut
c
Luciferase
CMV
EMCVwt
EMCVmut
NRF
HeLa
50%
X=1299
10%
X=126
0.6%
X=54
5%
X=34
d
HUVEC
54%
X=179
27%
X=54
6%
X=16
10%
X=11
e
PBL
48%
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<0.1%
7%
X=33
f
CD 34+ Progenitors
20%
X=1270
3%
X=27
0.2%
1.2%
X=20
Net Luminescence, A.U. x 10³
GFP
g
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3%
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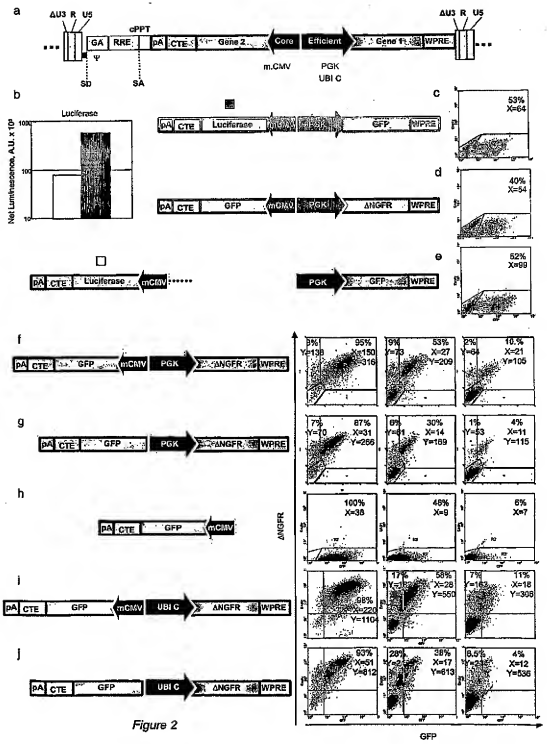
GFP
h
ΔNGFR
GFP
30%
Mean = 91
M1
M1
Events
ΔNGFR
GFP

Figure 1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ΔU3 R U5
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GA
RRE
pA
CTE
Gene 2
Core
Efficient
Gene 1
WPRE
ΔU3 R U5
Ψ
SD
SA
m.CMV
PGK
UBI C
b
Luciferase
Net Luminescence, A.U. x 10³
pA CTE Luciferase mCMV
c
53%
X=64
pA CTE Luciferase IRES GFP WPRE
d
40%
X=54
pA CTE GFP mCMV PGK ΔNGFR WPRE
e
52%
X=69
PGK GFP WPRE
f
pA CTE GFP mCMV PGK ΔNGFR WPRE
g
pA CTE GFP PGK ΔNGFR WPRE
h
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j
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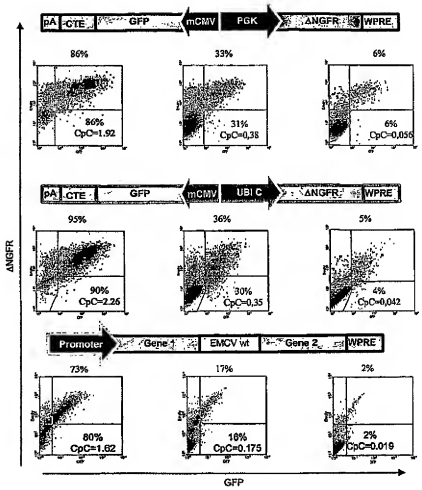
ΔNGFR
GFP

Figure 2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CTE
GFP
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PGK
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86%
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31%
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6%
6%
CpC=0,056
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CTE
GFP
mCMV
UBI C
ΔNGFR
WPRE
95%
90%
CpC=2.26
36%
30%
CpC=0,35
5%
4%
CpC=0,042
Promoter
Gene 1
EMCV wt
Gene 2
WPRE
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2%
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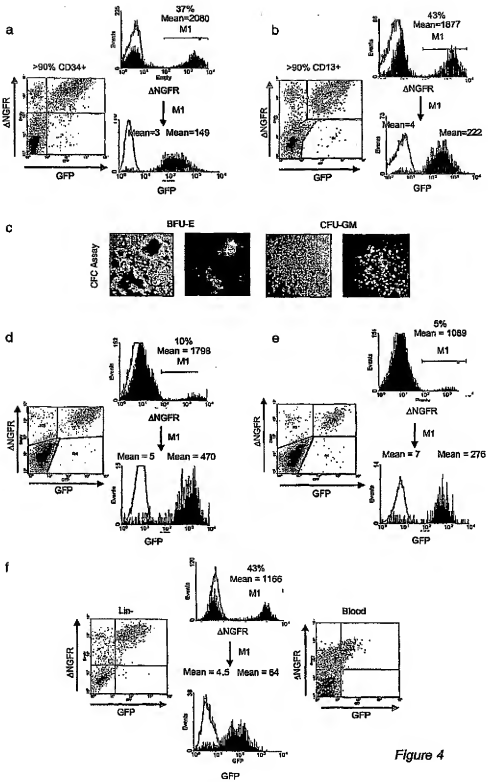
ΔNGFR
GFP

Figure 3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ΔNGFR
GFP
37%
Mean=2080
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M1
Mean=3
Mean=149
GFP
b
>90% CD13+
ΔNGFR
GFP
43%
Mean=1877
M1
M1
Mean=4
Mean=222
GFP
c
BFU-E
CFU-GM
CFC Assay
d
ΔNGFR
GFP
10%
Mean = 1798
M1
M1
Mean = 5
Mean = 470
GFP
e
ΔNGFR
GFP
5%
Mean = 1089
M1
M1
Mean = 7
Mean = 276
GFP
f
Lin-
ΔNGFR
GFP
43%
Mean = 1166
M1
M1
Mean = 4.5
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GFP
Blood
ΔNGFR
GFP

Figure 4

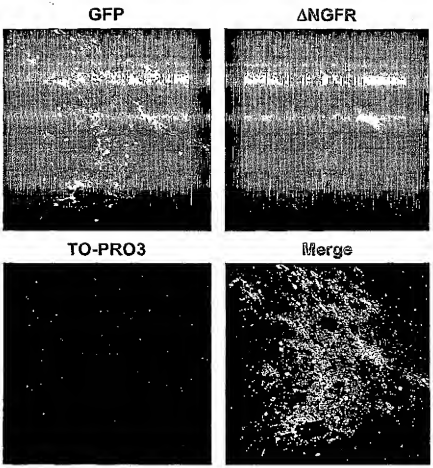
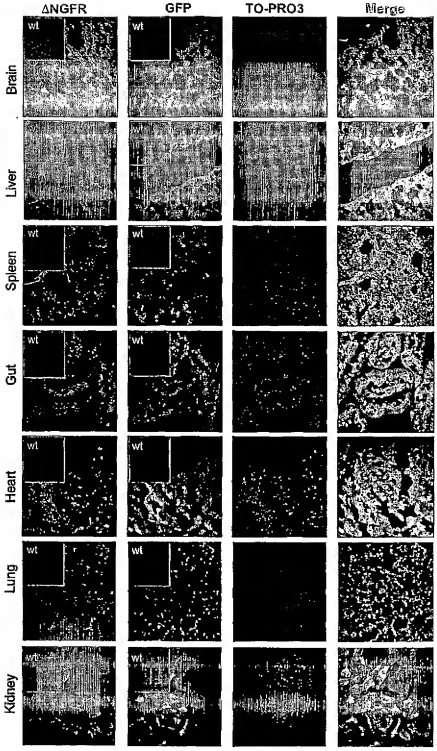


Figure 5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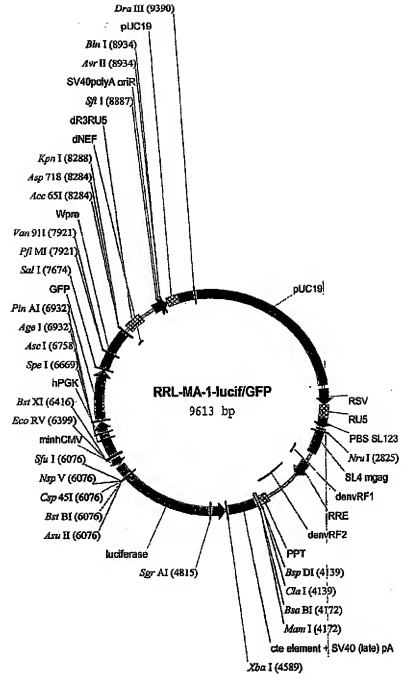
GFP
TO-PRO3
Merge
Brain
Liver
Spleen
Gut
Heart
Lung
Kidney
wt

Figure 6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Dra III (9390)
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dR3RU5
dNEF
Kpn I (8288)
Asp 718 (8284)
Acc 65I (8284)
Wpre
Van 91I (7921)
Pfl MI (7921)
Sal I (7674)
GFP
Pin AI (6932)
Age I (6932)
Asc I (6758)
Spe I (6669)
hPGK
Bst XI (6416)
Eco RV (6399)
minhCMV
Sfu I (6076)
Ntp V (6076)
Csp 45I (6076)
Bst BI (6076)
Azu II (6076)
luciferase
Sgr AI (4815)
Xba I (4589)
cte element + SV40 (late) pA
Mam I (4172)
Bsa BI (4172)
Cla I (4139)
Bsp DI (4139)
PPT
denvRF2
RRE
denvRF1
SL4 mgag
Nru I (2825)
PBS SL123
RU5
RSV
pUC19

Fig 7a

Fig. 7b (1 of 3)

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Fig. 7b (2of 3)

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Fig. 7b (3 of 3)

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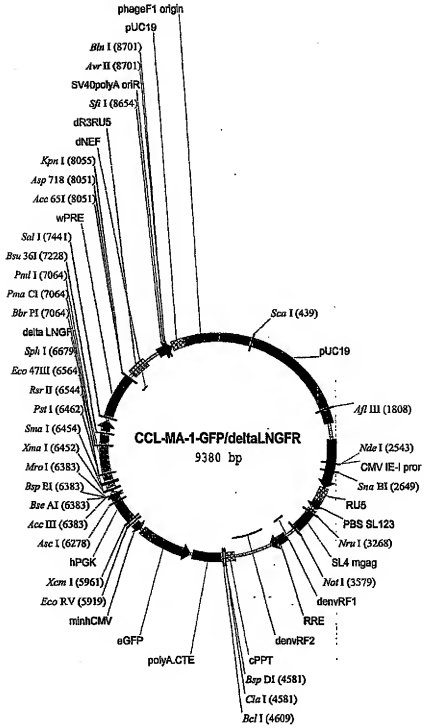
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Avr II (8701)
SV40polyA oriR
Sfi I (8654)
dR3RU5
dNEF
Kpn I (8055)
Asp 718 (8051)
Acc 65I (8051)
wPRE
Sal I (7441)
Bsu 36I (7228)
Pml I (7064)
Pma CI (7064)
Bbr PI (7064)
delta LNGF
Sph I (6679)
Eco 47III (6564)
Rsr II (6544)
Pst I (6462)
Sma I (6454)
Xma I (6452)
Mro I (6383)
Bsp EI (6383)
Bze AI (6383)
Acc III (6383)
Asc I (6278)
hPGK
Xcm I (5961)
Eco RV (5919)
minhCMV
eGFP
polyA.CTE
cPPT
Bsp DI (4581)
Cla I (4581)
Bcl I (4609)
denvRF2
RRE
denvRF1
SL4 mgag
Not I (3579)
Nru I (3268)
PBS SL123
RU5
Sna BI (2649)
CMV IE-I pror
Nde I (2543)
Afl III (1808)
pUC19
Sca I (439)
CCL-MA-1-GFP/deltaLNGFR
9380 bp

Fig. 8a

Fig. 8b (1 of 3)

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Fig. 8b (2 of 3)

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Fig. 8b (3 of 3)

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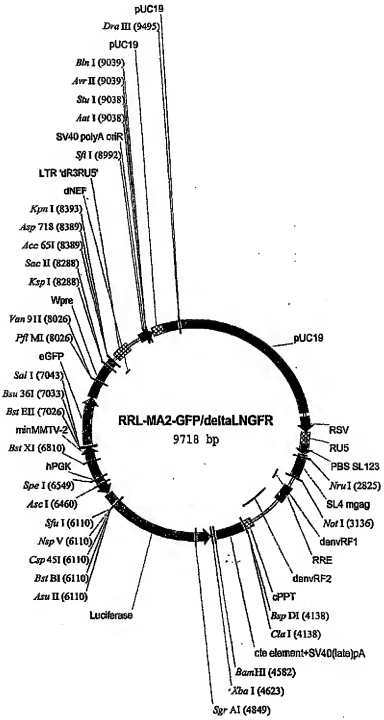
RRL-MA2-GFP/deltaLNGFR
9718 bp
pUC19
Dra III (9495)
pUC19
Bln I (9039)
Awr II (9039)
Stu I (9038)
Aat I (9035)
SV40 polyA cmR
Sfi I (8992)
LTR 'dR3R'U5'
dNEF
Kpn I (8393)
Asp 718 (8389)
Acc 65I (8389)
Sac II (8288)
Ksp I (8288)
Wpre
Van 91I (8026)
Pfl MI (8026)
eGFP
Sal I (7043)
Bsu 36I (7033)
Bst EII (7026)
minMMTV-2
Bst XI (6810)
hPGK
Spe I (6549)
Asc I (6460)
Sfu I (6110)
Nsp V (6110)
Csp 45I (6110)
Bst BI (6110)
Asu II (6110)
Luciferase
pUC19
RSV
RU5
PBS SL123
Nru I (2825)
SL4 mgag
Not I (3136)
denvRF1
RRE
denvRF2
cPPT
Bsp DI (4138)
Cla I (4138)
cte element+SV40(late)pA
BamHI (4582)
Xba I (4623)
Sgr AI (4849)

Fig. 9a

Fig. 9b (1 of 3)

caggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgaga
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Fig. 9b (2 of 3)

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Fig. 9b (3 of 3)

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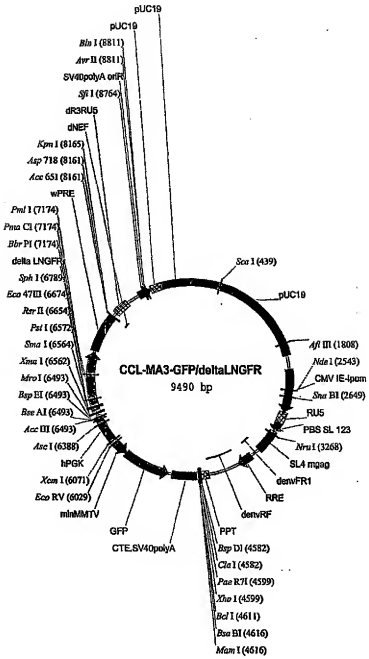
CCL-MA3-GFP/deltaLNGFR
9490 bp
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pUC19
Bla I (8811)
Avr II (8811)
SV40polyA oriP
Sfi I (8764)
dR3RU5
dNEF
Kpn I (8165)
Asp 718 (8161)
Ace 651 (8161)
wPRE
Pml I (7174)
Pma CI (7174)
Bbr PI (7174)
delta LNGFR
Sph I (6785)
Eco 47III (6674)
Rsr II (6654)
Pst I (6572)
Sma I (6564)
Xma I (6562)
Mro I (6493)
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Bse AI (6493)
Acc III (6493)
Asc I (6388)
hPGK
Xcm I (6071)
Eco RV (6029)
minMMTV
GFP
CTE.SV40polyA
PPT
Bsp DI (4582)
Cla I (4582)
Pac I/I (4599)
Xho I (4599)
Bcl I (4611)
Bse BI (4616)
Mam I (4616)
RRE
denvRF
denvFR1
SL4 mpag
Nru I (3268)
PBS SL 123
RU5
Sna BI (2649)
CMV IE-lpam
Nde I (2543)
Afl III (1808)
Sca I (439)
pUC19

Fig. 10a

Fig. 10b (1 of 3)

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Fig. 10 b (2 of 3)

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Fig. 10b (3 of 3)

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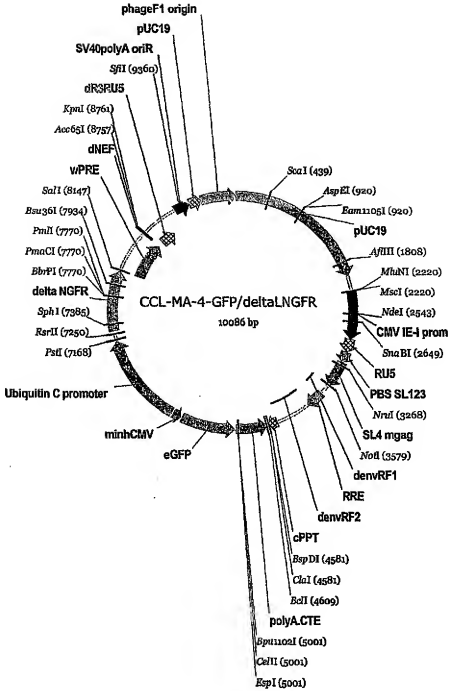
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KpnI (8761)
Acc65I (8757)
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wPRE
SalI (8147)
Bsu36I (7934)
PmlI (7770)
PmaCI (7770)
BbrPI (7770)
delta NGFR
SphI (7385)
RsrII (7250)
PstI (7168)
Ubiquitin C promoter
minhCMV
eGFP
CCL-MA-4-GFP/deltaLNGFR
10086 bp
ScaI (439)
AspEI (920)
Eam1105I (920)
pUC19
AflIII (1808)
MluNI (2220)
MscI (2220)
NdeI (2543)
CMV IE-I prom
SnaBI (2649)
RU5
PBS SL123
NruI (3268)
SL4 mgag
NotI (3579)
denvRF1
RRE
denvRF2
cPPT
BspDI (4581)
ClaI (4581)
BclI (4609)
polyA.CTE
Spu1102I (5001)
CelII (5001)
EspI (5001)

Fig. 11a

Fig. 11b (1 of 3)

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Fig. 11 b (2 of 3)

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Fig. 11b (3 of 3)

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